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ACQUISITION OF M PROTEIN BY PNEUMOCOCCI THROUGH TRANSFORMATION REACTIONS*

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Since the initial observations by Griffith (1) of the transformation of pneumococcal capsular types in the mouse, considerable progress has been made in elucidating the mechanism whereby this specific cellular alteration is effected. The studies of Avery, MacLeod, and McCarty (2) have shown that acquisition of the ability to produce a different capsular carbohydrate is dependent upon the application to unencapsulated pneumococcal cells of highly polymerized desoxyribonucleic acid. More recently, Boivin and his coworkers (3) have shown that an analogous transformation can be induced in *E. coli* with surface carbohydrate as the acquired character and have brought evidence that the agent responsible for the change is desoxyribonucleic acid. Weil and Binder (4) using culture filtrates, were able occasionally to produce type transformations of three strains of *Shigella paradysenteriae* but no information is available concerning the chemical nature either of the principle inducing the transformation or of the acquired character. To the present time, therefore, no clear evidence has been obtained of the transformation of bacterial cells with a protein as the acquired character.

In the present paper, transformation of pneumococci *in vitro* and *in vivo* with the acquisition of a different M protein (5) is reported. In addition, the independent variability of M protein and specific capsular polysaccharide is described.

Materials and Methods

Transformation Reactions.—The techniques employed in preparing transforming extracts and vaccines were those described by MacLeod and Krauss (6). Transforming extracts and vaccines prepared from pneumococcus strains I-SVI and III-A66 were used. Transformation reactions *in vivo* were carried out by the method of Griffith (1). For transformation reactions *in vitro*, the technique of Avery *et al.* (2) was employed with the following modification. When cultures of Dawson rough (7) variants on solid media were used as the source of inoculum, the tubes containing the transforming system were inoculated by means of a straight needle which had been touched to a peripheral portion of a colony. When pneumococci were grown in liquid medium, the inoculum was one drop of a 10^{-4} dilution of an 18 hour culture.

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Strains of Griffith Rough and Dawson Rough Pneumococci.—Pneumococcus strain II-R36NC (Griffith rough) and two derivatives of it were used as inocula in most of the experiments reported. One derivative strain, II-R36N1_{43}, was obtained by transferring strain II-R36NC daily for 43 transfers in broth containing 1 per cent absorbed antiserum directed against the M protein of the parent strain. Lancefield extracts of this strain, II-R36N1_{43}, gave only faint reactions with anti-M sera that reacted strongly with extracts of the parent strain, II-R36NC. Strain II-R36N1_{43} retains the colonial appearance characteristic of Griffith rough pneumococci. A Dawson rough variant of strain II-R36NC, selected according to Dawson's method (7), was also used. Plates of neopeptone-meat infusion agar containing 5 per cent defibrinated rabbit blood were inoculated from a broth culture of strain II-R36NC with a fine wire in such a manner that colonies were spaced at a distance of 0.5 to 1.0 cm. The plates were incubated for 7 to 14 days at 37°C. After 5 days, rough, flat, fan-like excrescences similar to those described by Dawson were noted at the periphery of some colonies. Portions of a number of these outgrowths were inoculated by means of a straight needle into tubes of neopeptone-meat infusion broth and incubated overnight. Only those broth cultures showing growth characterized by complete autoagglutination were used to inoculate a second series of plates. In Dawson's experience, cultures of the rough variants of pneumococci obtained by this process of selection showed a strong tendency to revert spontaneously to the Griffith rough colonial form when cultivated in nutrient broth (7). By repeated selection and inoculation from those colonies showing the least capacity for reversion, however, strains of Dawson rough organisms were obtained after 10 serial transfers which showed little or no tendency to revert spontaneously to the Griffith rough form even after 5 or 6 rapid serial transfers in a highly nutrient liquid medium. In Lancefield extracts of such relatively stable Dawson rough strains, the M protein characteristic of the parent culture could still be demonstrated, but it appeared reduced in amount. In view of Dawson's observation that reversion of colonial type is favored by transfer in nutrient broth, inocula of the Dawson rough strain R36ND_{11}, used in the present transformation experiments, were obtained by touching a needle to the desired portion of the colony on solid medium. This inoculum was then transferred directly to tubes containing the transforming system.

In addition to the pneumococcal strains described above, in certain experiments another Griffith rough variant, II-R36A, derived from the encapsulated type II strain II-D39S, was used as inoculum.

In the designation of transformed strains the following set of conventions has been used: the designation of the parent strain is followed by a dash, the letter M and an Arabic numeral to indicate the type of M protein, and the letter S and a Roman numeral to indicate the type of capsular carbohydrate possessed by the cell. For example, strain II-R36NC-M2′SIII is a strain of pneumococcus derived from capsular type II possessing the type-specific protein (M2′) of the parent type II cells and the capsular carbohydrate (SIII) of type III. Similarly, strain II-R36NC-M1SIII is one derived from capsular type II possessing the type-specific protein (M1) of type I and the capsular carbohydrate of type III (SIII).

Preparation of Anti-M Sera, M Extracts, and *Techniques of Precipitin and Agglutination Tests.*—The methods used were those described in the preceding paper (5).

EXPERIMENTAL

1. Transformation Reactions of Pneumococci with Acquisition of Capsular Polysaccharide but without Acquisition of M Protein.—Because pneumococcus strains II-R36NC and II-R36A had been shown previously to be susceptible to transformation reactions involving the acquisition of capsular polysaccharides, these two Griffith rough variants of pneumococcus II-D39S (capsular type II)

were selected for study. The results of *in vitro* transformation of these two strains to capsular type III and of strain II-R36A to an intermediate variant of capsular type I are shown in Table I. Although capsular polysaccharides have been acquired, the type-specific protein remains that of the parent strain, demonstrating the independent variability of the two cellular components. Strain II-R36A-M2′SI(int) is of further interest because of its low virulence for mice. Pneumococci of this intermediate strain, possessing a very small, but detectable capsule of type I polysaccharide, kill mice only when amounts of 16 hour culture as large as 1×10^{-1} to 1×10^{-2} cc. are injected. In view of the fact that this strain was derived from strain II-D39S which is fully virulent for mice (M.L.D. = 1×10^{-8} cc. of a 16 hour broth culture) and because both

TABLE I

Antigenic Composition of Pneumococcus Strains II-R36NC and II-R36A and of Derivative Strains Obtained by Transformation Reactions in Vitro

Strain	Source of transforming extract strain	M protein	Capsular SSS
II-R36NC	—	2′	None
II-R36NC-M2′SIII	III-A66	2′	III
II-R36A	—	2′	None
II-R36A-M2′SIII	III-A66	2′	III
II-R36A-M2′SI(int)	I-SVI	2′	I

strains possess the same M protein, the findings suggest that the M protein of pneumococcus plays a minor rôle in the determination of virulence. This observation is in accord with the one noted in the preceding paper that anti-M sera afford little or no protection against infection with homologous encapsulated organisms.

2. Transformation in Vitro of Pneumococci with Concomitant Acquisition of M Protein and of Capsular Carbohydrate.—In none of the experiments performed *in vitro* with strains II-R36NC or II-R36A was the acquisition of M protein demonstrated. Inasmuch as these two strains possess M2′ protein, this finding may not be altogether remarkable. Although transformation of pneumococci of one capsular type in the mucoid or encapsulated phase directly to the mucoid phase of another capsular type has been reported (8), it seems probable that the transformation occurred because of the appearance during growth of unencapsulated mutants derived from the original mucoid strain. The cells of these unencapsulated variants, lacking presumably the characters requisite for the production of capsular polysaccharide of any type are susceptible to transformation upon exposure in a suitable environment to desoxyribonucleic acids from the homologous or heterologous types. When transformation takes place, the altered cells again produce capsular carbohydrate, the type being determined by the source of the nucleic acids.

It was reasoned by analogy, therefore, that if it were possible to select cells deficient in M protein, these cells might be capable of acquiring this cellular component through the transformation reaction. To obtain cells deficient in M protein, pneumococcus II-R36NC was grown in neopeptone broth containing 1 per cent of a potent anti-M2′ protein rabbit serum which had been absorbed with strains I-192R and III-A66R2 to remove species-specific antibodies. Lancefield extracts of cells obtained from the sixteenth daily transfer in antiserum broth and of cells from the same culture transformed to capsular type III showed still the original M2′ protein. Extracts of cells from the thirty-fourth transfer showed, however, an apparent reduction in the amount of M2′ protein produced by the culture, though its presence was still detectable. When cells from the forty-third transfer, strain II-R36N1_{43}, were tested in transformation reactions with an extract of strain III-A66, colonies were obtained on subculture which were of capsular type III. Of two colonies tested, one gave rise to a strain possessing type 2′M protein. The cells from the other colony, however, were found on subculture to possess type 3 M protein as well as type III SSS. The parent cell of this culture had undergone apparently, a double transformation with acquisition of SSS III and M3 protein.

An alternative method for obtaining cells deficient in M protein was sought by growing rough variants of pneumococcus II-R36NC according to the method of Dawson described in the section on methods. Transformations to capsular type III of Dawson rough variants from serial transfers 3, 5, and 8 showed no concomitant acquisition of M3 protein. On the eleventh serial transfer of the Dawson rough variant derived from strain II-R36NC, examination of Lancefield extracts made from these cells suggested a diminution in the quantity of M2′ protein produced by the culture. Cells from several colonies of the eleventh passage, strain II-R36ND_{11}, were tested in transformation reactions with an extract made from pneumococcus III-A66. Following incubation at 37°C. for 24 hours, samples were plated on blood agar. Examination of these platings revealed three colonial forms: mucoid, Griffith rough, and Dawson rough. Control cultures showed only the Dawson rough colonial phase initially and after 6 serial subcultures. Griffith rough and mucoid colonies from several cultures were selected and grown for the preparation of Lancefield extracts. Extracts of cultures of most of the mucoid colonies (capsular type III) showed M2′ protein. One mucoid colony, however, gave rise to a culture possessing M3 protein in addition to the capsular polysaccharide of type III. It is of interest that a Griffith rough strain recovered from the same culture contained M2′ protein although it had undergone alteration in colonial morphology from the Dawson rough form.

By two independent methods of selection, therefore, strains of pneumococcus were obtained which are capable of acquiring through transformation reactions

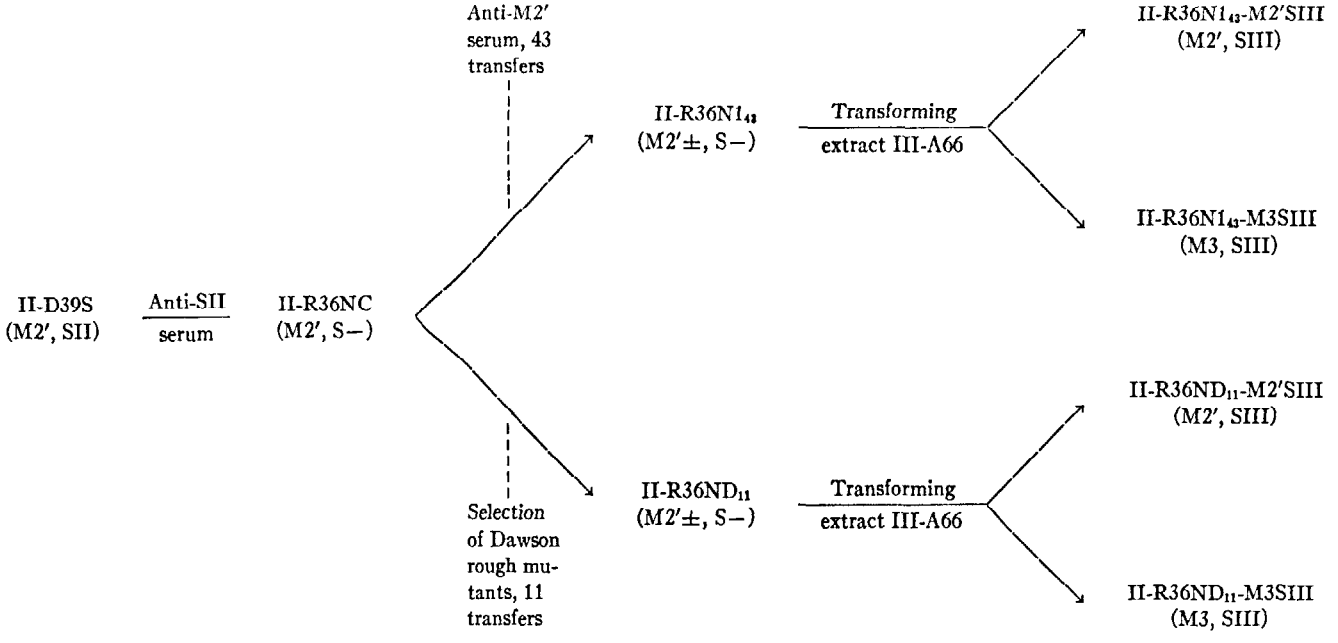
in vitro, a type specific M protein different from that possessed by the original type II strain from which they were derived. The results of these experiments are summarized schematically in Fig. 1 together with an antigenic analysis of the strains concerned.

3. Transformation of Pneumococci in Vivo, with Concomitant Acquisition of M Protein and of Capsular Polysaccharide.—Transformation of pneumococci with acquisition of M protein was accomplished *in vivo* by the method of Griffith. Mice were inoculated subcutaneously with a live culture of the Griffith rough strain, II-R36NC, together with a heat-killed vaccine of encapsulated pneumococci prepared by the method of MacLeod and Krauss (6). In none of the experiments were viable organisms detected in the transforming vaccines, either by mouse inoculation or by culture. Transformation of pneumococcus II-R36NC to capsular type I was induced by a vaccine prepared from pneumococcus I-SVI in eight of ten mice inoculated. Transformation to capsular type III was effected with a vaccine of pneumococcus III-A66 twice in ten attempts. In one instance of transformation to each capsular type, concomitant acquisition of M protein occurred. The strains in which the acquisition of two new characters took place differed distinctively in their colonial morphologies from those of the strains from which the transforming vaccines were made, whereas capsular transformations of pneumococcus II-R36NC possessing either the M protein of that strain or the M protein of the transforming vaccine were indistinguishable from each other in their colonial appearance. In view of these morphological observations and the absence of viable organisms from vaccines, there can be no doubt that a different M protein can be acquired through transformation carried out *in vivo* by the technique of Griffith.

4. Combination of Three Inheritable Characters within a Single Pneumococcal Strain.—To determine whether a strain of pneumococcus possessing the colonial properties of one type and the M protein of a second type could be transformed so that it now produced the capsular polysaccharide of a third type, the following experiments were undertaken. Pneumococcus strain II-R36NC-M1SI, which had acquired M1 protein and SSSI through transformation *in vivo* of pneumococcus II-R36NC in the presence of a transforming vaccine of pneumococcus I-SVI, was grown in 20 per cent antipneumococcus capsular type I serum broth. A Griffith rough variant containing M1 protein was obtained after eight transfers. This variant was then exposed *in vitro* to a transforming extract of pneumococcus III-A66. Platings of this culture revealed encapsulated pneumococci which on analysis showed the basic colonial morphological properties of strain II-R36NC, the type 1 M protein of strain I-SVI, and the type III capsular polysaccharide of strain III-A66. It is possible, therefore, by transforming reactions to combine three inheritable characters of three antigenically distinct pneumococci within a single cell. (Fig. 2.)

FIGURE 1

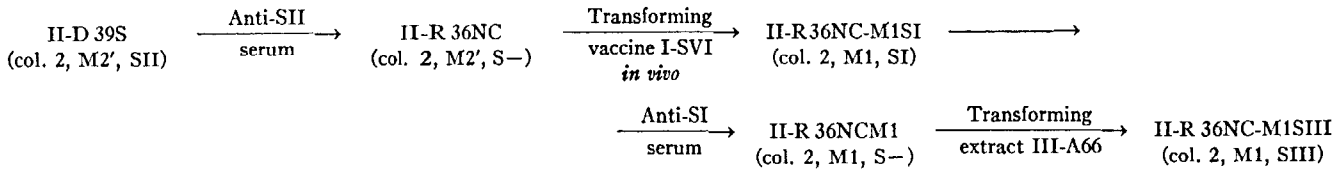
Methods of Selecting Rough Variants of Pneumococcus Strain II-D39S and of Directing the Acquisition of New Specific Capsular Polysaccharide and M Protein by Means of Transformation Reactions in Vitro



Antigenic analysis of strains is contained in parentheses below strain designations. The type-specific protein is indicated by the letter M and the Arabic numeral following it, the capsular polysaccharide by the letter S and Roman numeral following it. Absence of the antigen is indicated by −, a culture containing probably an increased proportion of deficient cells by ±. Notations above and below the arrows indicate the method used in selecting variants and the source of transforming extracts.

FIGURE 2

Combination of Inheritable Characters of Three Pneumococcal Strains within a Single Strain



The legends used are the same as those employed in Fig. 1. The retention of basic colonial morphological properties (autolytic and hemolytic) of the parent strain in the ensuing transformations is indicated by the inclusion of the symbol col. 2 in the parentheses below the strain designations.

DISCUSSION

The studies described in this paper demonstrate that the M protein of pneumococcus can be acquired through the transformation reaction and add a new class of biological compounds, the presence and type specificity of which are subject apparently to the control of highly polymerized desoxyribonucleic acids. Transforming extracts are capable of causing the acquisition, not only of the capsular polysaccharide of pneumococcus as described previously by others, but also of the M protein and would appear capable also of inducing alteration of colonial morphology from the Dawson rough to the Griffith rough variant when the appropriate conditions exist. Inasmuch as it has been demonstrated that pneumococcus capsular carbohydrate and M protein may vary independently of one another, the results obtained suggest that transforming extracts of encapsulated pneumococci contain a multiplicity of desoxyribonucleic acids which control the specificities of the several cell characters described.

At the present time, transformation of pneumococci involving acquisition of M protein cannot be carried out with as high a frequency as can that of the capsular polysaccharides. Although acquisition of a new M protein by pneumococcus strain II-R36NC occurs occasionally in the mouse under the influence of heat-killed transforming vaccines, acquisition of a new M protein by this strain *in vitro* has not been observed. It is possible, however, that cultures of strain II-R36NC contain cells which are deficient in M protein but in such small numbers that detection of transformation is unlikely unless strongly selective factors operate. In a sense, acquisition of a new M protein by pneumococcus strain II-R36NC *in vivo* is analogous to the transformation *in vitro* of pneumococci of one capsular type directly to another capsular type reported by Dawson and Warbasse (8). It was their opinion that although unencapsulated forms were not seen in the cultures, transformation took place probably by way of these variants. If, however, pneumococcus II-R36NC is grown in the presence of anti-M2′ serum or if Dawson rough variants are selected by suitable cultural methods, strains are obtained by either technique which, when extracted by hot acid solutions, yield less M protein than the parent strain. It seems probable that the methods of selection employed have increased the relative number of M protein–deficient cells in the culture and have improved the probability of detection of cells with a newly acquired M protein following exposure of the culture to transforming extracts. This hypothesis would account for the presence of capsular transformations associated with either the M protein of the cells used as transforming agents or that of the culture subjected to transformation. That only a fraction of the cells exposed to transforming extract acquire a new M protein is not remarkable if it be recalled that in all probability but a small percentage of M protein–containing, unencapsulated cells similarly treated undergo capsular type transformation.

The experiments reported here suggest that acquisition of a class of proteins by pneumococci is probably controlled by desoxyribonucleic acids. Of the transformations reported previously, all analyzed chemically have concerned the acquisition of polysaccharides. Although transformation involving the surface carbohydrate of *E. coli* (8) and of the type-specific antigens of *Sh. paradysenteriae* (4) have been accompanied by altered fermentative activities, the alterations suggest the loss of enzymes rather than their acquisition.

The demonstration of the independent variability of capsular polysaccharide and of M protein in transformed pneumococci is of interest in view of the observation in the preceding paper (5) that the same capsular polysaccharide may be associated with different M proteins in nature, and that the same or very similar proteins may occur with different capsular polysaccharides. An additional finding of interest is the fact that by the use of transformation reactions it is possible to combine within a single pneumococcal cell characters derived from three distinct pneumococcal strains. If it be assumed that the number of type-specific M proteins and of distinctive colonial forms approximates the number of known capsular types, then the possible number of antigenically distinct pneumococci is in excess of 500,000. The demonstration that it is possible to effect such combinations of antigens in the laboratory and the observations made upon naturally occurring strains suggest the desirability, whenever possible, of isolating chemically the reactive substances which enter into an antigenic analysis of bacteria in order to avoid or to elucidate "cross-reactions" which may prove confusing. That such a procedure is useful is borne out by the studies of the relations of the M and T antigens of group A β-hemolytic streptococci (9).

SUMMARY

Acquisition by pneumococcal variants of M protein and of capsular polysaccharide different from those present in the parent strain has been effected *in vitro* by means of transforming reactions with extracts of heterologous encapsulated pneumococci. Similar transformations have been accomplished *in vivo* with heat-killed vaccines as the transforming agents.

Independent variation of pneumococcal capsular polysaccharide and M protein observed in nature can be brought about also in the laboratory. By means of transforming reactions, it has been demonstrated that inheritable characters of three distinct pneumococcal strains can be combined within a single strain.

It is suggested that acquisition of M protein through transformation reactions occurs in cells deficient in that character.

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